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The primary cilium influences interleukin-1β-induced NFκB signalling by regulating IKK activity

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ABSTRACT

The primary cilium is an organelle acting as a master regulator of cellular signalling. We have previously shown that disruption of primary cilia assembly, through targeting intraflagellar transport, is associated with muted nitric oxide and prostaglandin responses to the inflammatory cytokine interleukin-1β (IL-1β). Here, we show that loss of the primary cilium disrupts specific molecular signalling events in cytosolic NFκB signalling. The induction of cyclooxygenase 2 (COX2) and inducible nitrous oxide synthase (iNOS) protein is abolished. Cells unable to assemble cilia exhibit unaffected activation of IκB kinase (IKK), but delayed and reduced degradation of IκB, due to diminished phosphorylation of inhibitor of kappa B (IκB) by IKK. This results in both delayed and reduced NFκB p65 nuclear translocation and nuclear transcript binding. We also demonstrate that heat shock protein 27 (hsp27), an established regulator of IKK, is localized to the ciliary axoneme and cellular levels are dramatically disrupted with loss of the primary cilium. These results suggest that the primary cilia compartment exerts influence over NFκB signalling. We propose that the cilium is a locality for regulation of the molecular events defining NFκB signalling events, tuning signalling as appropriate.

1. Introduction

Primary cilia influence a wide variety of cell signalling pathways linked to development, health and disease. These include established functions for primary cilia in left–right determination [1], mechanosensation [2,3], defined roles in the regulation of hedgehog (Hh) [4,5], platelet derived growth factor (PDGF) signalling [6], and more recently, wnt [7–10], insulin growth factor (IGF) [11], and transforming growth factor (TGF) [12] signalling. The early hypotheses and interpretations were that the cilium acted as an antenna for signal detection, which, in many contexts, has proven to be the case. Increasingly, however, research indicates that the specialized ciliary compartment often influences signal transduction, downstream of the reception of an initial stimulus but upstream of transcriptional responses. This may tune not only the magnitude of signalling, as exemplified by roles in hedgehog and wnt where transcriptional activity can be both promoted and dampened [10,13], but also the temporal characteristics of signalling as exemplified by IGF receptor sensitization [11]. In doing so the cilium encodes the necessary additional complexity where one stimulus activates a plethora of targets.

Much of our understanding of primary cilia biology, including these examples, comes from studying the effect of mutations to genes encoding the cilium's specialized trafficking machinery. This machinery includes proteins that function in intraflagellar transport (IFT) which is responsible for anterograde and retrograde molecular trafficking up and down ciliary microtubules. Mutations in proteins thought to support these processes are the source of a growing number of human pathologies known collectively as ciliopathies. In 2012 we used a hypomorphic mutation of the gene *tg737*, encoding for polaris or IFT88, to understand the relevance of primary cilia elongation in response to the inflammatory cytokine interleukin-1β [14]. Cilia length change is a function of altered ciliary trafficking and contents and as such indicative of potential alterations to cilia function for example in wnt [15], hedgehog signalling [16] and mechanotransduction [17] contexts. However, a function for the cilium in IL-1β signalling was previously unheralded. Near complete loss of the ciliary compartment, as a result of this mutation or pharmacological blockade of cilia elongation, yielded a diminished response at the level of nitrite release and prostaglandin E_2 release [14]. These inflammatory second messengers are the products of inducible nitrous oxide synthase (iNOS) and a cascade of phospholipid metabolism steps culminating with stimulation of the rate limiting enzyme cyclooxygenase 2 (COX2)

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and ultimately prostaglandin E synthases (PGES). The purpose of the present study was to unravel the mechanism that accounts for the loss of this inflammatory response when the ciliary trafficking is compromised.

Interleukin-1β activates a wide range of intracellular signalling leading to induction of many alterations in cellular activity. To a large extent IL-1β pro-inflammatory activities are modulated by either NFκB signalling, or mitogen activated kinase (MAP) activities [18] or combinations of both. NFκB signalling components are well-conserved, activated in response to a plethora of stimuli and regulate and/or are entwined in many cellular processes. These include immunity and differentiation among many others. For a recent review see [19]. A rapid cascade of protein–protein interactions defines the pathway. Phosphorylations, SUMOlyations and ubiquitinations, both positively and negatively regulate signalling. Two such events are at the heart of pro-inflammatory signalling, namely inhibitor of kappa kinase (IKK) mediated phosphorylation [20] of the protein inhibitor of NFκB (IκB) [21] and the subsequent proteosomal destruction of IκB following ubiquitination by FWD1 [22]. This allows NFκB transcription factor subunits to be released to the nucleus [23,24] where they activate or co-activate many targets including iNOS and COX2 [25–28]. This key sequence of events is preceded by receptor-linked events including the molecular binding between and activations of interleukin receptor associated kinases (IRAKs), tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6), and transforming growth factor activated kinase 1 (TAK1). TAK1 is ultimately responsible for IKK activation through phosphorylation of the IKK complex regulatory subunit NEMO [29]. Complex formation between these upstream effectors, namely IRAK1 and TRAF6 and TAK1 and NEMO, facilitates pathway activation [30]. Heat shock proteins (HSP) are known to both facilitate and control many of the cascade of molecular interactions throughout signal transduction. One such HSP is the chaperone Hsp27, proposed to regulate TRAF, TAK, JNK, p38 and IKK activities [31–36].

2. Materials and methods

2.1. Cell culture

The current study used murine cells harbouring a hypomorphic mutation in *TG737* encoding for IFT88 as described previously [14,37]. Once removed from conditional immortalization these cells have a primary chondrocyte phenotype [37]. Chondrocytes are an apt model for this project as they express high prevalence of primary cilia expressed relatively parallel to culture surface on the basal lateral surface, an advantage for confocal imaging. In addition there is biological relevance as interleukin-1β is found elevated in the joints during many forms of arthritis [38] as are the inflammatory chemokines PGE_2 and NO [39–41]. The cell line was cultured in low glucose DMEM supplemented with 10% foetal calf serum, 88 U mL^{-1} penicillin, 90 μg·mL streptomycin and 2.5 mM L-glutamine. Immortalization, under permissive conditions of 33 °C culture and in the presence of 10 ng·mL^{-1} Interferon-γ (INF) (R&D systems, UK), was used only for maintenance of the line. For all experiments cells were cultured at 37 °C without INF for 3 days, as has previously been shown to be sufficient to switch off SV40 expression, before use as primary cells. For protein expression studies cells were cultured on plastic, and for immunofluorescence studies cells were cultured on FCS coated glass coverslips.

2.2. Protein isolation

For total cell protein isolations cell lysates were collected quickly on ice. Plastic culture wells were washed once in ice cold phosphate buffered saline (PBS) with 50 μM sodium orthovanadate. Subsequently an ice cold lysis buffer of PBS, a cocktail of protease inhibitors (Roche), 50 μM sodium orthovanadate (Sigma) and 0.1% Igebal (Sigma) was added and samples were left on ice for 15 min before scraping and 5 homogenization steps, through a 21 G needle. Samples were then spun at 13,000 RPM for 15 min at 4 °C before supernatant was frozen in liquid nitrogen, stored at −80 °C. Where required nuclear and cytoplasmic fractions were collected using a protocol based on an isolation kit (EpiSeeker extraction kit ab113474, Abcam, UK). In short, after a gentle initial lysis protocol the cytoplasmic fraction was removed and a buffer containing dithiothreitol or DTT was added to the leftover nuclear pellet to create a highly concentrated nuclear fraction lysate which was incubated on ice for 15 min with vortexing before refrigerated centrifugation and storage at −80 °C.

2.3. Western blotting

For western blotting lysate samples were diluted 1:1 with Laemmli buffer then boiled at 100 °C for 5 min. For total protein studies samples of around 30 μL, or 50 μg protein as assessed by the Bradford assay, were run on a 10 or 12% tris(hydroxymethyl)aminomethane–hydrochloride gels before transfer to a nitrocellulose membrane. Nuclear fraction lysates were more concentrated and only 10 μL volume was required but again evenly loaded following Bradford analysis. Transfers and loading were checked using ponceau staining. 1 h in 5% milk or 3–5% BSA (for blocking) preceded primary antibody incubations overnight at 4 °C. Licor infrared secondary antibodies were incubated at 1:15,000 (anti-mouse and anti-goat) or 1:7500 (anti-rabbit) for 1 h at room temperature preceded and followed by 3 × 10 min washes in 0.1% PBS Tween. Relative protein expression was established by quantitative analysis of specific bands (LI-COR Odyssey integrated intensity values). Linearity of western blots analysis was tested by constructing a standard curve using serial dilutions of samples probed for β-tubulin. Relative expression was quantified relative to β-actin, β-tubulin or lamin C. Antibodies used were: Rabbit mAb to COX2 1:1000 (Cell Signaling 12282), Rabbit pAb to iNOS 1:500 (Abcam ab3523), Rabbit pAb to NFκB p65 1:1000 (Abcam ab7970), Rabbit mAb to phospho IKK ser176/180 1:500 (Cell Signaling 2697), Mouse mAb to phospho IκBα ser32/36 1:500 (Cell Signaling 9246), Rabbit mAb to IκBα 1:10,000 (Abcam ab32518), Mouse aAb to β-actin 1:10,000 (Abcam ab8226), Goat pAb to lamin A/C 1:2000 (Santa Cruz sc-6215), Mouse mAb to β-tubulin 1:5000 (Sigma T4026), Goat pAb to hsp27 (Santa Cruz SC-1048), and Rabbit mAb to phsp27 ser82 1:1000 (Cell Signaling 9709).

2.4. NFκB p65 binding ELISA

Nuclear lysates were assessed to quantify relative nuclear p65 transcript binding with a kit (ab133128) from Abcam, UK. In short, a specific double stranded DNA sequence containing the NFκB response element is immobilized onto the wells of a 96 well plate for ELISA analysis of p65 binding as detected by antibody binding and HRP conjugate detection. Both positive and non-specific binding controls were included. Absorbance was measured at 450 nm. Results were normalized to lamin C expression.

2.5. Immunofluorescent staining and imaging

Samples prepared for immunofluorescent labelling were fixed for 7 min at 37 °C in 4% paraformaldehyde. Glass coverslips were treated with 0.5% triton for 5 min and blocked with 5% goat serum for 30 min. Primary incubations of either 1:500 Rabbit anti-NFκB p65, 1:2000 mouse anti-acetylated alpha tubulin (Sigma), 1:1000 rabbit anti-arl13b (source Bioscience), or 1:500 goat anti-hsp27 (Santa Cruz sc-1048) were conducted at 4 °C for at least 16 h before washing in 0.1% bovine serum albumin in phosphate buffered saline. Alexa secondary antibodies (Life Technologies, UK) were used at 1:500 or 1:1000, and incubated for 1 h at room temperature. Secondary only controls were used for image thresholding but non-specific secondary binding was very limited. For co-localization of hsp27 an hsp27 peptide (also Santa Cruz-sc1048p) was used for competitive binding with primary

antibody prior to cell incubations to ensure specific binding of the primary antibody. All primary antibody binding has also been previously confirmed to be product specific as assessed by western blot. For p65 translocation studies and for co-localization studies glass coverslip samples were imaged using a confocal microscope (Leica SP2) with a x63/1.05 NA objective and a pixel size of 0.1 × 0.1 μm or smaller. p65 translocation images are reconstructed maximum projection images created using Image J software as previously described [14,15]. Confocal slices were taken with a step size ≤0.5 μm.

2.6. Data analysis and statistics

Primary cilia prevalence was assessed from multiple fields and multiple cell seeding preparations to avoid bias. Differences were assessed by Chi-squared tests. Cilia length was assessed similarly but using Mann Whitney tests as length data failed normality testing. COX2 and iNOS expression was compared using 2-way ANOVA with post Bonferroni testing. For other protein expression work comparisons between two groups and for fold changes unpaired Student's t-tests were used. ELISA data used two-way ANOVA with post-testing. Data analysis and testing were all conducted in GraphPad prism5.

3. Results

3.1. Hypomorphic mutation to IFT88 inhibits the induction of COX2 and iNOS expression in response to IL-1

We set out to determine the specific nature of the influence the cilium exerts using the cell model that first highlighted a role for the cilium in IL-1 signalling. The Oak Ridge Polycystic Kidney (ORPK) mutation reduces cilia prevalence (Fig. 1A/B) as we have previously reported [14,37,42]. In the present study cilia prevalence was 10% in ORPK compared with 80% in WT, a statistically significant difference ($p < 0.05$, Chi squared test n = 345 and 319 cells from 11 fields for each group, data in

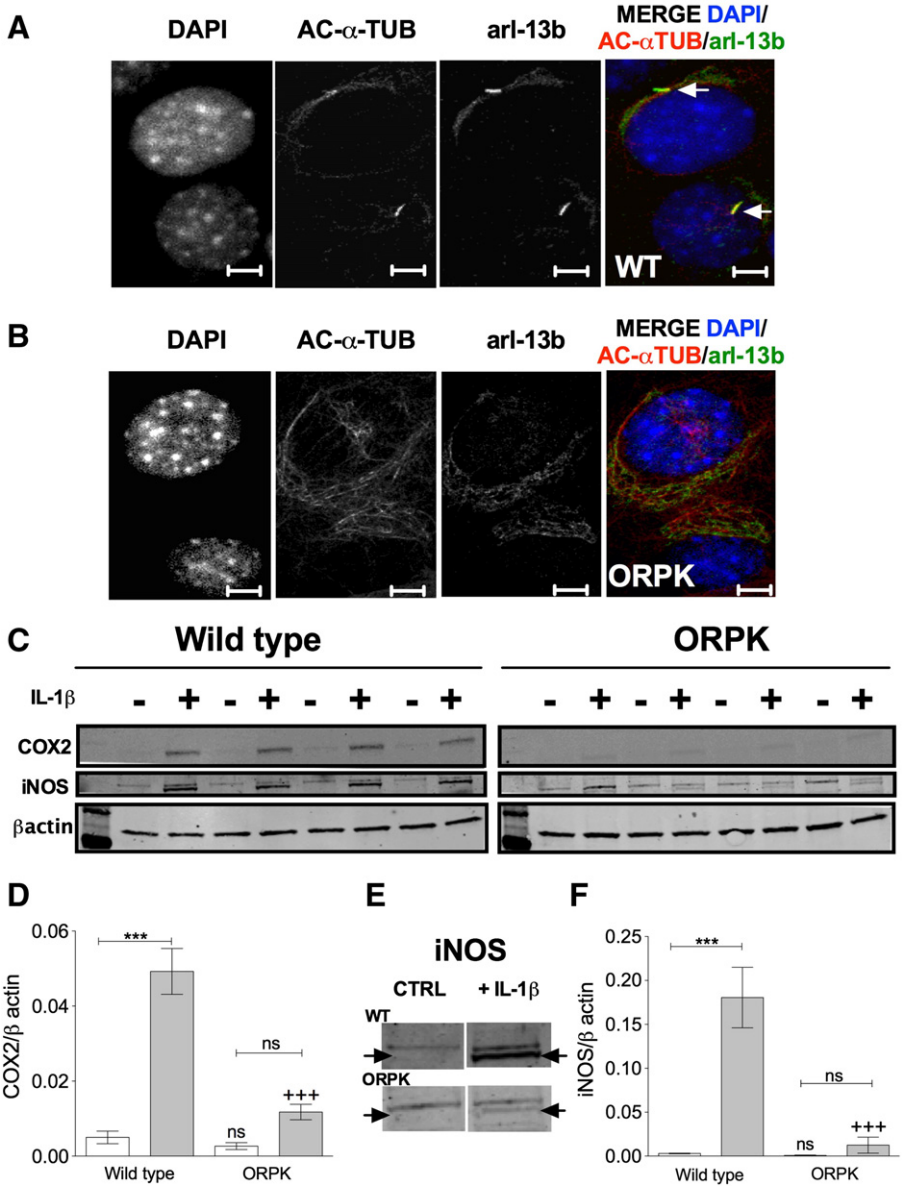

Fig. 1. Interleukin-1β activation of COX2 and iNOS expression is inhibited in cells unable to elaborate the primary cilia structure as a result of dysfunctional IFT. **A/B** Immunofluorescent labelling of cilia markers indicates loss of primary cilia in ORPK. Cilia in WT marked by arrows. Scale bar 10 μm. **C** Western blots indicate robust increases in COX2 and iNOS observed in WT are lost in ORPK (4 replicates for both WT and ORPK). **D** Quantification of relative COX2 protein levels in WT and ORPK treated with IL-1β (10 ng·mL^{-1} for 24 h). Error bars are S.E.M., n = 4. **E** Enlargement to clarify iNOS band (lower band) used for quantification. **F** Quantification of relative iNOS protein levels in WT and ORPK treated with IL-1β (10 ng·mL^{-1} for 24 h). Error bars are S.E.M., n = 4.

supplementary material S1, Fig. A). Where primary cilia are still observed cilia length was statistically significantly reduced in ORPK cells compared with WT cells (Supplementary material S1, Fig. B). A median length of 2.2 μm was measured in WT, 1.0 μm in ORPK ($p < 0.001$, Mann Whitney U test, n = >200 cilia per group). The induction of COX2 and iNOS was assessed at the protein level in WT and ORPK cells in order to establish whether the cilium regulates upstream signalling, gene/protein induction or enzyme activity. After 24 h treatment with IL-1β (10 ng·mL^{-1}) WT cells exhibited statistically significant up-regulations of both COX2 and iNOS protein levels ($p < 0.0001$, 2-way ANOVA, n = 4, Fig. 1C/D/F). In stark contrast ORPK cells showed no such large increases in COX2 or iNOS, such that treatment with IL-1β had no statistically significant effect on either protein levels (2-way ANOVA, n = 4, Fig. 1C/D/F). COX2 and iNOS protein levels in ORPK cells treated with IL-1β were statistically significantly different to that in WT also treated with IL-1β ($p = <0.0001$, 2-way ANOVA, n = 4). This concurs with our previous data for the enzyme products, PGE_2 and NO [14].

3.2. Loss of the primary cilium is associated with altered NFκB p65 translocation to the nucleus

One prominent signalling cascade upstream of the induction of COX2 and iNOS is the NFκB cascade, defined largely by the translocation of the p65 transcription factor subunit from the cytoplasm to the nucleus. From immunofluorescent (IF) imaging of NFκB p65 in WT cells it was clear that translocation of p65 took place within 5 min of IL-1β treatment and was established after 20 min (Fig. 2A). Comparing ORPK with WT cells no apparent differences were seen before IL-1 treatment (Supplementary material S2). However, it appeared that nuclear translocation of the p65 subunit was delayed from initiation in ORPK. After 5 min of IL-1 treatment, ORPK cells displayed limited nuclear intensity (Fig. 2B, top panels). Nuclear intensity was reduced at 20 min in ORPK, compared with WT (Fig. 2B, bottom panels). To quantify total and compartmentalized protein expression western blotting was used. It was apparent that baseline total p65 expression levels were higher in ORPK cells than WT but expression was also highly varied in ORPK ($p = 0.18$, unpaired Student's t-test, n = 6, Fig. 2C). Expression was highly varied in ORPK (coefficient of variation 107% compared with 29% in WT cells). Nuclear fractions were assessed for p65 protein in control preparations and following 20 min IL-1β exposure. Whilst statistically significant enrichment of the nuclear fraction was observed in WT ($p = 0.025$, unpaired Student's t-test, n = 7) no such statistically significant change was seen in ORPK, in concord with observations from IF (Fig. 2D). The transcript binding capability of p65 protein in these nuclear fractions was assessed by means of an enzyme linked competitive binding assay using a plate coated with DNA response element for p65. This indicated reduced transcript binding capability in lysates from ORPK treated for 20 min with IL-1β with respect to those from WT cells ($p = 0.045$, 2-way ANOVA, n = 6). Taken together this data

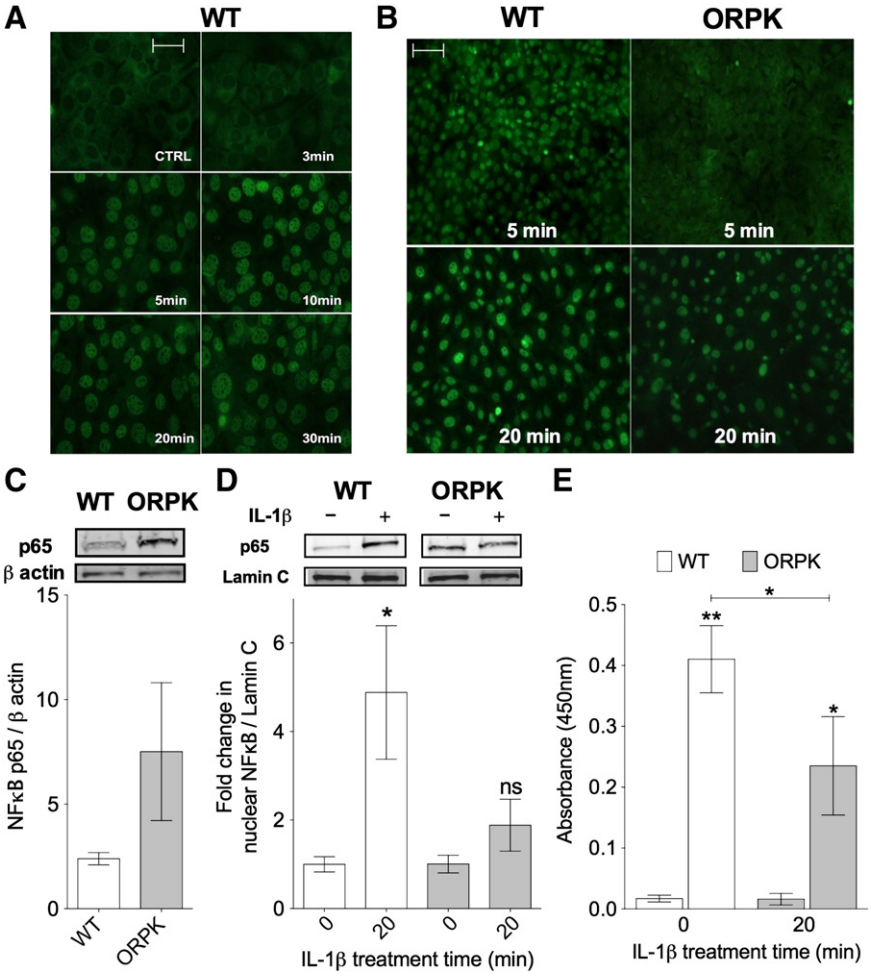

Fig. 2. The translocation and transcript binding of NFκB p65 subunit is inhibited in cells unable to elaborate the primary cilia structure. **A** Immunofluorescent labelling of p65 subunit in WT cells treated with IL-1β for stated periods of time. Scale bar 50 μm. **B** Lower power microscopy images of immunofluorescent labelling of p65 subunit in WT cells and ORPK cells treated with IL-1β for stated periods of time. Scale bar 150 μm. **C** Western blot analysis of total p65 expression relative to β-actin in WT and ORPK cells (no IL-1β treatment). **D** Relative nuclear expression of p65 normalized to lamin C expression. Expression levels shown as fold increases with 20 min IL-1β (10 ng·mL^{-1}) compared with control. **E** Relative p65 DNA transcript binding for nuclear fractions isolated from WT and ORPK cells before and after 20 min IL-1β (10 ng·mL^{-1}). For all quantifications shown, error bars are S.E.M.

implies that loss of the cilium is associated with diminished NFκB p65 translocation to the nucleus and DNA binding at like for like time points.

3.3. The primary cilium influences IKK-mediated phosphorylation of IκBα and its subsequent destruction

NFκB p65 is held in the cytoplasm by binding to inhibitor of κB (IκB). The key events regulating the release of the p65 subunit were assessed by western blots at appropriate time points. Following IL-1 pathway activation the IκBα molecule is phosphorylated, prior to its ubiquitination and proteosomal destruction. This phosphorylation event is carried out by inhibitor of kappa kinase or IKK which itself is activated by phosphorylation as a result of upstream signalling events. In WT cells this activating phosphorylation of IKK was observed at 10 min post IL-1 exposure by western blot using an antibody that recognizes serine phosphorylations to the activation loops of both catalytic subunits of IKK namely α and β (Fig. 3A). In ORPK this event is unaffected. No phosphorylation of IKK was observed at later time points in either WT or ORPK cells. Expression of phosphorylated IKK with IL-1 treatment is not significantly different in WT and ORPK (Fig. 3B).

In contrast, IKK activity, as assessed by western blot analysis of pIkB, is diminished in ORPK (Fig. 4A, top blot) such that phosphorylation of IκBα at 10 min is statistically significantly reduced ($p < 0.001$, 2-way ANOVA, $n = 3$) with respect to WT, as quantified in Fig. 4B. The degradation of IκB from 10 min onwards (Fig. 4A, second blot down) is incomplete in ORPK with respect to WT cells such that even by 30 min IκBα expression remains in ORPK cells. Indeed when IκBα expression is quantified there was no statistically significant reduction in expression compared to control preparations prior to IL-1 treatment in ORPK cells (Fig. 4C). Importantly, whilst control expression levels varied, as seen for p65, there was no statistically significant difference in IκBα amounts between WT and ORPK cells before IL-1 treatment (Supplementary material S3). These data indicate the cilium to be important specifically to IKK activity and downstream Iκβ degradation. Upstream receptor and cytosolic signalling is unaffected by loss of the cilium.

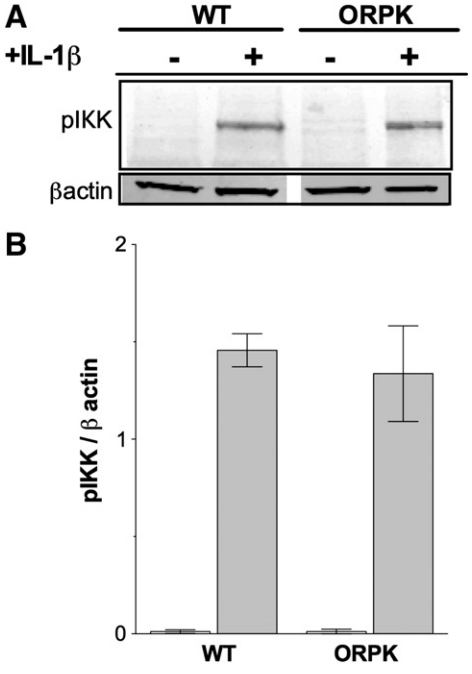

Fig. 3. Loss of cilium has no effect on IKK activation. **A** Example blot for pIKK expression in WT and ORPK cells treated with IL-1β (10 ng·mL^{-1}) for 10 min. **B** Quantification of pIKK expression relative to β-actin. Data is presented as S.E.M., $n = 3$.

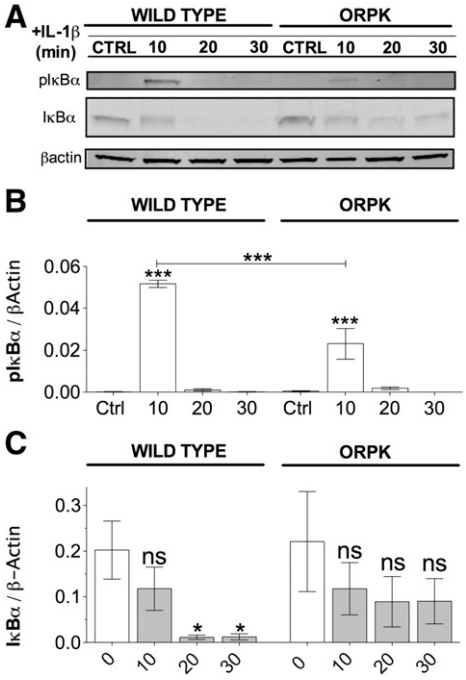

Fig. 4. IKK activity and IκBα destruction are diminished with loss of the primary cilium. Example western blots and quantifications of key signalling events assessed during the activation of IL-1β signalling at time points following the addition of IL-1β (10 ng·mL^{-1}). **A**. Expression of pIκBα, total IκBα, and β-actin was assessed in control and at 10, 20 and 30 min time points. **B** Quantification of pIκBα expression relative to β-actin. **C** Quantification of IκBα expression with time after IL-1β treatment is also normalized to β-actin. For all quantifications and S.E.M. are shown, $n = \geq 3$.

3.4. Immunofluorescence confocal imaging reveals ciliary localization of IKK regulator, hsp27

The primary cilium conventionally regulates cell signalling by means of trafficking key components through the ciliary compartment or into the ciliary membrane. This includes receptors, molecules involved in receptor activation, molecules key to the downstream transduction of signalling, and transcription factors. We therefore investigated the location of signalling molecules involved in this part of the pathway by immunofluorescent staining (IF). WT cells were used for western blotting, both in control experiments but at all the time points of IL-1 treatment and earlier time points such as 1, 3 and 5 min after IL-1 treatment. Previously, IκBα has been found to associate with the microtubular organizing centre [43]. In this study it was throughout the cell (Fig. 5A) (including sometimes on the cilium (Fig. 5A enlarged panel)), although not clearly enough to be considered enriched above that of the general cytosolic compartment and therefore confidently attributed to the ciliary compartment. From translocation studies no clear evidence for ciliary enrichment of p65 was observed. pIKK expression was difficult to observe by IF presumably due to low amounts but in samples treated with IL-1β at timepoints from 3 to 5 min, apparent weak pIKK staining was observed. In about 15% of cells this staining was co-localized to the base of the cilium (Fig. 5B). Previously, the primary cilium has been linked to the regulation of expression of the chaperone hsp27 [44], observed in motile cilia [45] and proposed to be directly influential over IKK associated signalling events, including by direct interaction [31–35]. We find hsp27 expression to be highly enriched in the cilium above cytoplasmic levels. Staining was also co-localized with the cilia localized GTPase arl13b (Fig. 5C/D), although not when arl13b was elsewhere in the cell. In a few cases (<5%) apparent cilia localized hsp27 staining was not intense or homogenous through the axoneme (Supplementary material S4). In this example hsp27 staining is more clearly observed throughout the rest of the

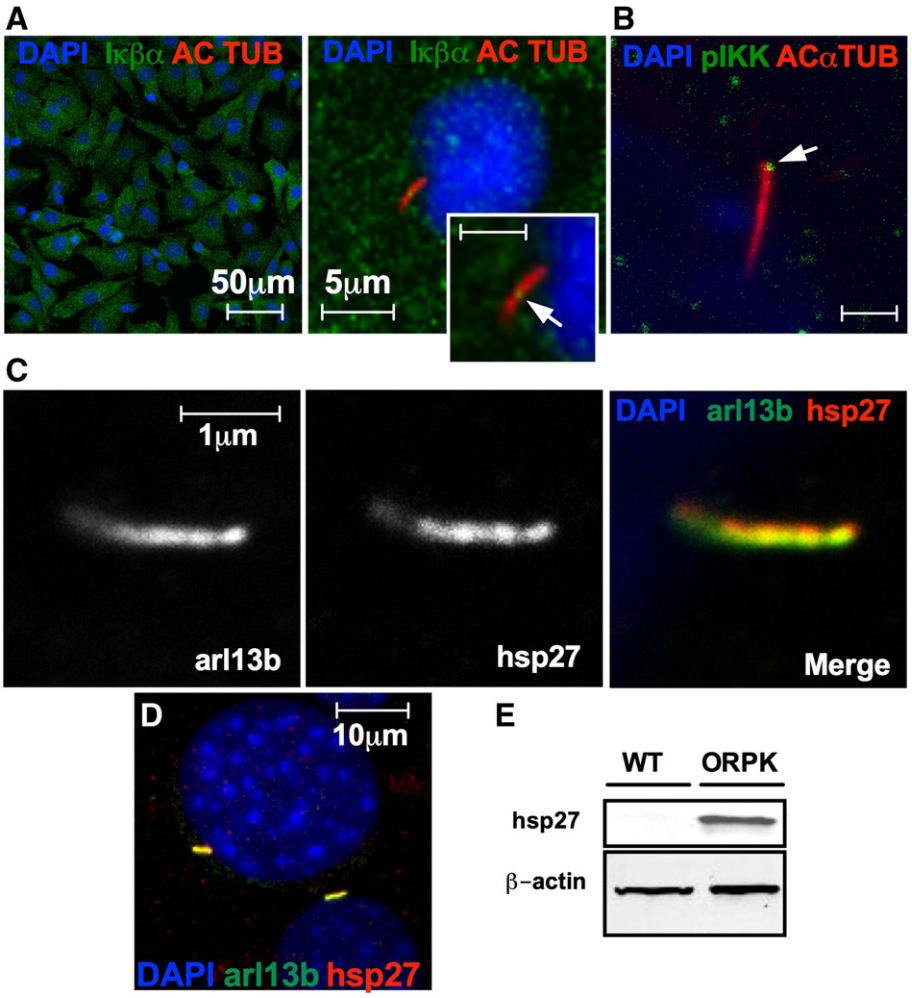

Fig. 5. IKK regulator hsp27 is enriched in the primary cilium. **A** Immunofluorescent (IF) labelling indicates possible cilia localization of IκBα as indicated by arrow, at 10 min after IL-1β treatment. However, staining in cilium was no higher than prevalent staining in the wider cytoplasm. Scale bar in enlargement is 2.5 μm. **B** Example of basal body localization of pIKK staining, also indicated with arrow. Scale bar is 2.5 μm. **C** IF labelling shows striking co-localisation of hsp27 to arl13b staining in the primary cilium. Images from single confocal slice. **D** Maximum Z projection shows majority, but not all hsp27 signal in primary cilia. **E** Western blot analysis of total hsp27 indicates far greater levels of hsp27 in ORPK cells such that hsp27 and phsp27 expression is barely detectable by western blot.

cytoplasm but hsp27 staining was always observed in the cytoplasm when 3D maximum projections of the cells were created (Fig. 5D). When cellular expression of hsp27 was compared between WT and ORPK cells hsp27 expression was predictably very low in WT, such that the band is barely visible when compared with the markedly higher expression in ORPK (Fig. 5E). Likewise when the phosphorylation of hsp27 with IL-1 treatment was assessed this was only observable in western blot in the ORPK cells (Supplementary S5). It was not possible to image phsp27 by IF.

4. Discussion

This study develops previous findings describing loss of responses to IL-1β in cells where primary cilia trafficking is impaired [14]. Previously, we did not observe normal increases in the inflammatory markers PGE_2 and NO in cells where IFT was impaired. This implied that the ciliary compartment is required for these inflammatory responses. In the present study we show that these findings were likely due to primary cilia influence over the magnitude and timing of upstream NFκB pathway signalling events.

Whilst the primary cilium has well-established roles in the regulation of many signalling pathways, beyond our previous study of 2012, there is little to link the cilium to the regulation of IL-1β signalling. However, low NO has been a clinical marker for some time in a human genetic disorder, primary ciliary dyskinesia. This disorder relates to dysfunction of motile cilia, which share much structural homology with primary cilia. In addition there is evidence that cells isolated from these patients have a reduced capacity to respond to infectious challenge [46]. Moreover, very recently, human disorders related to primary cilia dysfunction, including Jeune syndrome [47] and short rib polydactyly (SRP) syndrome type III [48] have been linked to WDR34, already linked to NFκB signalling [49]. Recent studies have linked NFκB signalling components to motile [50] ciliogenesis and primary cilia disassembly in stem cells [51].

Our present study indicates that the cilium exerts influence at the bottleneck in the NFκB signalling cascade. Loss of the cilium influences events after early receptor-linked events but before protein induction and activation. PGE_2 and NO are products of the induction of the phospholipid-modifying enzyme PGES and iNOS respectively. In the case of PGE_2, COX2 is the rate-limiting enzyme induced. Removal of the cilium inhibits IL-1-induced expression of COX2 and iNOS, in accordance with a failure of upstream signalling, rather than deregulated transcription. Membrane located, receptor-linked signalling and immediate pathway activation appears unaffected by loss of the cilium but cannot be ruled out. IKK subunit phosphorylation, governed by the activities of TAK1, was not significantly altered in ORPK cells. This is also supported by the observation of IL-1-induced phosphorylation of hsp27 in ORPK cells as this is also downstream of TAK1 activities albeit

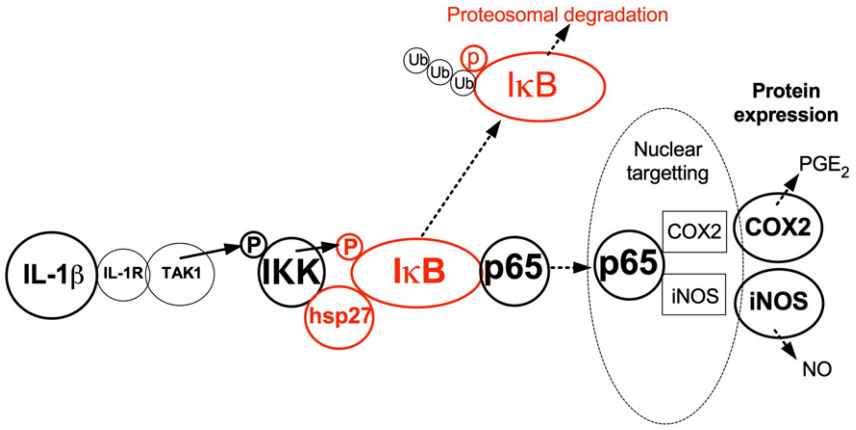

Fig. 6. A simplified signalling schematic to show proposed events where the cilium is proposed to regulate (highlighted in red). In the absence of IL-1, IκB traps p65 in the cytoplasm. In the presence of IL-1, receptor related events lead to the activation (phosphorylation as denoted by small black p) of IKK. These events are not dependent upon the ciliary compartment, as indicated by data in Fig. 3. However, the primary cilium may represent a critical location for, even scaffold upon which, the key molecular interactions between activated IKK and IκB, leading to IκB destruction, take place (as indicated by the data in Fig. 4). These events allow NFκB p65 release to the nucleus and are mistimed or diminished in magnitude without the cilium (as indicated by the data in Fig. 2). Ciliary trafficking of IKK and IκB to interact with ciliary hsp27 (Fig. 5) may allow controlled and rapid phosphorylation of IκB and its subsequent destruction, releasing a burst of p65 transcription factor to bind to transcriptional targets such as iNOS and COX2. In the absence of functional IFT88 cilia trafficking (ORPK) the delay or reduction in magnitude of these events results in a failure to induce target (Fig. 1).

via p38 pathway activation. The focus therefore moved to the activities of cytoplasmic IκB, which exerts inhibitory influence by holding the NFκB transcription subunit p65 away from the nucleus.

The cilium and its trafficking appear to be critical specifically to the phosphorylation of IκB by IKK, and therefore subsequent IκB destruction which is dependent upon this IKK activity. When the cilium is lost IκB levels are abnormally sustained, where expression is quickly abolished in WT cells. There is, predictably, strong concord between these data and downstream p65 nuclear translocation. Nuclear p65 when assessed qualitatively by immunofluorescent staining following IL-1 exposure, was still observed, even with loss of the cilium, but is diminished in magnitude and appears delayed. This is to be expected, given the sustained expression of IκB. This was supported by analysis of nuclear fractions quantifying p65 levels and DNA binding capacity. These inhibitions are not of such dramatic magnitude as those seen ultimately downstream at the level of iNOS, COX2 and their products, implying a few possibilities. One, that the cilium also exerts wider influence over other components of IL-1 signalling including p38 MAP kinases — this is the subject of a current, ongoing separate study. Secondly, it's is also important to note that the population of ORPK cells still assemble basal bodies, and 1 in 10 elaborates primary cilia, albeit stunted in nature. Finally, the perceived incomplete inhibition of IKK activity, p65 translocation and transcript binding may reflect a subtle, but vital, tuning role for the primary cilium. The cilium may govern the timing of these events and as such downstream this influence may materialize to a greater extent. The timing of NFκB signalling is of fundamental importance to downstream pathway activity [52] and specificity [53,54]. Aberrant pathway status and deregulated feedback on the pathway may underlie the large variation of baseline p65 and IκB expression in ORPK cells. All these data agree on multiple levels that the primary cilia compartment and its trafficking are of importance to and exert influence over NFκB signalling.

Previous studies have indicated alterations to primary cilia trafficking and contents during the activation of IL-1β signalling [14,42]. With this in mind we checked for the ciliary localization of the molecular components of the pathway. Despite broad cytoplasmic expression, we did find incidences of apparent but weak IκBα staining in the cilium. It must be said that this was not above the cytosolic levels and as such it's hard by microscopy alone to be certain of the ciliary localization of such protein. Predictably, even with IL-1 treatment, phosphorylated IKK staining was of low intensity but there was apparent ciliary base staining for this activated form of IKK, albeit weak. Therefore is again difficult to, with certainly, conclude that ciliary trafficking is taking place in response to IL-1. This will require further work and high resolution microscopy. The heat shock proteins function as chaperones to aid protein–protein interactions through protein folding. One such chaperone, hsp27, has been observed in motile cilia [45] and hsp27 expression is linked to that of the ciliary protein IFT88 [44]. Hsp27 has also been illustrated to both positively and negatively regulate NFκB signalling through interactions throughout the signalling cascade. More specifically, hsp27 and IKK are physical binding partners [55] and hsp27 directly alters IKK activity, although there is conflict, likely cell type specific, as to whether it plays a promotional or inhibitory role [31–35,56]. In the current study we find highly enriched hsp27 expression in the primary cilium, co-localized to the ciliary trafficking coordinator arl13b. We tentatively propose the cilia compartment to be a location for the highly regulated and precisely timed molecular interactions NFκB signal transduction requires, and that the presence of the chaperone hsp27 may underpin the molecular mechanism for this in NFκB signalling. The alteration to hsp27 location and total levels, as a result of dysfunctional ciliogenesis, may be the cause for inhibited NFκB signalling and downstream cellular responsiveness. These studies both illuminate the cilium's role and define the specific molecular events which the cilium potentially houses and thereby governs. This is summarized within the context of a highly simplified signalling cascade in Fig. 6, with events that are ciliary regulated in red.

The primary cilium is an ever-emerging exciting location for signalling cross-talk. It activities thereby influence the development, physiology and pathology of many tissues. In cartilage, mechanical and inflammatory cues drive arthritis. Interestingly, both cues alter primary cilia length [14,57], function and down-regulate IKK activities [58]. This may represent an anti-pathological feedback mechanism. This is of high relevance not only to cartilage but also in much wider contexts including cancers, where inflammatory and mechanical cues mediate pathology. Cilia localized heat shock proteins such as hsp27 have established roles in inflammatory signalling in chondrocytes [59,60] and hsp27 expression is altered in OA [61]. Hsp27 is a putative clinical marker in cancers [62–64]. Intriguingly hypoxia inducible factor 2 (HIF2), hugely influential to cell physiology and pathology is also known to bind and regulate IKK [65]. Recently, we have shown HIF2 to traffic through the cilium possibly in order to target its regulated destruction [42]. Many biochemical signals, for example increased cAMP, regulate ciliary trafficking and thereby structure and function [17] and also block downstream targets of inflammatory signalling [66]. This

strengthens the possibility that targeting cilia architecture may provide clinically useful modulation of cell behaviour by tuning the molecular events taking place on the cilium.

5. Conclusions

To conclude, this study reveals the mechanism for how the primary cilium influences inflammatory signalling, for the first time highlighting an influence over NFκB signalling and at the level of IKK activity. IKK and NFκB are influential in biological contexts spanning differentiation to disease. The specialized and therefore the potentially targetable primary cilia structure and machinery are now of worthy investigation in contexts where therapeutic regulation of IKK or NFκB signalling is sought.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2014.04.004.

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